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Delivery of Methylene Blue and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate from cross-linked poly(vinyl alcohol) hydrogels: A potential means of photodynamic therapy of infected wounds

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ABSTRACT

Poly(vinyl alcohol)–borate complexes were evaluated as a potentially novel drug delivery platform suitable for in vivo use in photodynamic antimicrobial chemotherapy (PACT) of wound infections. An optimised formulation (8.0% w/w PVA, 2.0% w/w borax) was loaded with 1.0 mg ml^{-1} of the photosensitisers Methylene Blue (MB) and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP). Both drugs were released to yield receiver compartment concentrations (>5.0 μg ml^{-1}) found to be phototoxic to both planktonic and biofilm-grown methicillin-resistant *Staphylococcus aureus* (MRSA), a common cause of wound infections in hospitals. Newborn calf serum, used to simulate the conditions prevalent in an exuding wound, did not adversely affect the properties of the hydrogels and had no significant effect on the rate of TMP-mediated photodynamic kill of MRSA, despite appreciably reducing the fluence rate of incident light. However, MB-mediated photodynamic kill of MRSA was significantly reduced in the presence of calf serum and when the clinical isolate was grown in a biofilm. Results support the contention that delivery of MB or TMP using gel-type vehicles as part of PACT could make a contribution to the photodynamic eradication of MRSA from infected wounds.

1. Introduction

Wound infection may be defined as the entry, growth, metabolic activity and resulting pathophysiological effects of a microorganism upon patient tissue. Infection has been shown to impair wound healing for both acute and chronic wounds. *Staphylococcus aureus* and coagulase-negative *Staphylococci* are the bacteria most frequently isolated from the deep tissue of chronic wounds with *S. aureus* reported to be present in up to 43% of infected leg ulcers and in up to 88% of non infected leg ulcers [1].

Bacterial wound infections are treated currently using topical antibiotics, such as mupirocin, fusidic acid and neomycin or systemic antibiotics, such as the β-lactams, macrolides and metronidazole, or a combination of both. However, bacterial resistance to all of these agents has been reported widely and numerous antibiotic-resistant bacteria now exist, including methicillin-resistant *S. aureus* (MRSA) which is the primary pathogen isolated from antibiotic resistant, nosocomial wound infections [2]. The increasing resistance of wound infections to both systemic and topical antibiotics has made effective treatment more difficult and accordingly, interest has arisen in the development of new treatment regimens.

Photodynamic antimicrobial chemotherapy (PACT) is defined as a medical treatment by which a combination of a sensitising drug and visible light causes selective destruction of microbial cells through the generation of singlet oxygen [3]. The ability of a light and drug combination to kill microorganisms has been known for over 100 years [4]. However, it is only recently with the beginning of the search for alternative treatments for antibiotic-resistant pathogens, that the phenomenon has been investigated in detail. As singlet oxygen is a non-specific oxidising agent and, hence, can have multiple cellular targets, it is widely thought that resistance to PACT is unlikely to develop. In addition, the drugs used in PACT exhibit selectivity for microbial cells over human cells that makes the treatment an attractive option. Numerous studies have shown PACT to be highly effective in the in vitro destruction of both Gram-positive and Gram-negative bacteria [5,6]. Indeed, a number of studies have shown that both planktonic and biofilm-grown MRSA can be killed efficiently using PACT [7–9]. However, reports on in vivo tests of PACT are scarce with the only studies that exist relating to investigations using periodontal

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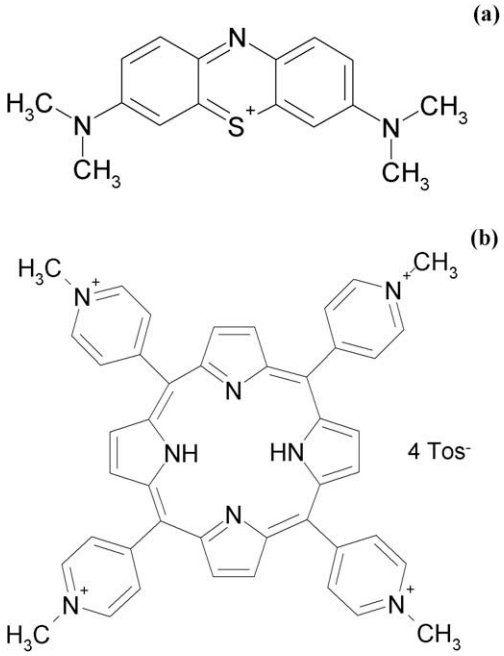

Fig. 1. The chemical structures of (a) Methylene Blue and (b) meso-tetro (N-methyl 1-4-pyridyl) porphine tetra tosylate (TMP).

administration of photosensitisers to dogs [10,11] and topical application of photosensitiser solutions to mice [12–14].

Our group has considerable experience in the design and clinical application of sophisticated drug delivery devices for both photodynamic therapy (PDT) of neoplastic disease [15] and topical drug delivery using cross-linked poly(vinyl alcohol) (PVA) hydrogels [16]. Recently, we have extended our interests to the formulation of systems for use in PACT [17–19]. Consequently, the aim of the present study was to evaluate PVA hydrogels as a delivery platform for model photosensitiser substances in the possible PACT of wound infections. The photosensitisers chosen were two cationic photosensitisers from different chemical classes, namely Methylene Blue (MB) and meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP), the structures of which are shown in Fig. 1. These photosensitiser-loaded hydrogels should be capable of conforming to the shape and contours of a wound, but maintain structural integrity whilst in place. They should release photosensitiser efficiently in a reasonable time frame and be capable of inducing antimicrobial activity following suitable illumination.

2. Materials and methods

2.1. Chemicals

Methylene Blue (MB), Poly(vinyl alcohol) 98–99% hydrolyzed, average M_W 31,000–50,000 (PVA), newborn calf serum (USA origin, sterile-filtered, cell culture grade) and sodium tetraborate (borax) were purchased from Sigma Aldrich, Dorset, UK. Meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP) was purchased from Frontier Scientific Europe Ltd., Carnforth, Lancashire, UK. Plastisol® medical grade poly(vinyl chloride) (PVC) emulsion, containing diethylphthalate as plasticiser, was provided by BASF Coatings Ltd., Clwyd, UK. All other chemicals used were of analytical reagent quality.

2.2. Microorganisms

A clinical MRSA isolate (MRSA 180) cultured from the skin of a hospitalised patient was used as the test organism in this study. The isolate was maintained on Mueller–Hinton Agar (MHA) slopes at 5 °C prior to use.

2.3. Preparation and characterisation of PVA–borate hydrogels

PVA, dissolved in deionised water, was added to a sodium tetraborate solution to give a final hydrogel comprising 20% w/w and 8% w/w of polymer and cross-linker, respectively. Defined amounts of MB or TMP were added to the borax solutions prior to addition to the PVA solutions to produce drug-loaded variants. Evaluation of the mechanical properties, both before and following photosensitiser addition, was performed using a TA-XT2 Texture Analyser (Stable Micro Systems, Haslmere, UK) in texture profile analysis (TPA) mode. Formulations were placed in poly(propylene) containers (circular diameter 44.0 mm, depth 55.0 mm) (Sarstedt©, Wexford, Republic of Ireland) with any air bubbles in the gels removed upon standing at ambient temperature (>6.0 h) prior to investigation. The tubular probe (10.0 mm in diameter and 150.0 mm in length) was compressed twice into each sample to a depth of 15.0 mm at a rate of 10.0 mm s^{-1}, with a 15.0 s delay between compressions. Four replicate measurements were made in each case at ambient temperature. Hardness and compressibility, which have previously been used to define the mechanical properties of hydrogels, were derived from the force–time plots produced by the TPA analysis [20].

The adhesive properties of the photosensitiser-loaded hydrogels were evaluated using the Texture Analyser in adhesive mode. Freshly excised (<2.0 h post slaughter) porcine skin was obtained from a local abattoir and used within 4 h. The excised skin was cut along the subcutaneous–dermal interface in order to separate the fatty layer from the epidermis and dermis. The epidermal side of the excised skin was then attached to a Perspex® block (2.0 cm^2) with cyanoacrylate adhesive, subsequently exposing the dermal side for analysis. This block was then fixed to the titanium base plate of the Texture Analyser using stainless steel screws. For analysis of dermal adhesion, the probe was moved down until intimate contact was achieved between the dermal layer and the hydrogel surface. Approximately 30.0 g of each hydrogel was loaded into a Perspex® well (circular diameter 60.0 mm, depth 15.0 mm), which was placed below the dermal substrate. Once contact was achieved, the interface was maintained for 30 s with a downward force of 0.1 N. After 30 s the probe moved upwards at a speed of 10.0 mm s^{-1}. Four replicate measurements were again made in each case at ambient temperature. Adhesion was defined as the force maxima of the force–time plot produced during detachment of the dermal substrate from the hydrogel surface [21].

2.4. Influence of newborn calf serum on hardness

The ability of PVA–borate hydrogels to maintain structural integrity upon mixing with serum was evaluated using the Texture Analyser in TPA mode. Newborn calf serum was used as a model of wound exudates. Hardness was the parameter measured to indicate any deviation in the integrity of the hydrogels upon treatment with fresh newborn calf serum. Hydrogels were loaded, as before, into poly(propylene) containers. Different volumes (5.0, 10.0 and 20.0 ml) of fresh newborn calf serum were poured onto the hydrogels. To mimic the clinical situation more closely, serum was not stirred into the hydrogels. The serum was left in place for defined time periods (30, 45, 60, 120 and 360 min) and then removed and the hardness of the hydrogels determined. Four replicate measurements were again made in each case at ambient temperature.

2.5. Drug release studies

One-dimensional drug release was evaluated using modified cell culture inserts (Nunc® No. 137508, Nalgene Nunc International, Rochester, USA; 25 mm diameter × 10 mm height) constructed with an integral poly(carbonate) membrane of 8.0 μm pore size, as described by Loughlin et al. [16]. Briefly, a defined mass of the hydrogel (4.0 g) with a photosensitiser loading of 1 mg ml^{-1} (MB or TMP) was added to these bespoke inserts and submerged into a release medium (100 ml), which was continuously stirred at 250 rpm. A phosphate buffer (BP 1999, pH = 6.8) was used as the receiver phase to mimic the slightly acidic environment of an infected or inflamed wound [22]. Sink conditions were maintained throughout the release experiment by ensuring that the total drug concentration in the receiver phase never exceeded 10% of its maximum solubility in the chosen buffer. At defined time intervals, 5.0 ml of receiver phase was removed, replaced by fresh buffer and the photosensitiser concentration determined spectrophotometrically at 290 nm (MB) or 425 nm (TMP) (Carey® 50 scan UV–Visible spectrophotometer, Varian Analytical Instruments Palo Alto, CA USA). In vitro release studies were carried out at ambient temperature (25 °C) and human body temperature (37 °C). Each release experiment was performed three times.

2.6. Light diffusion studies

Light must be capable of penetrating biological fluids present at a wound site if it is to excite the delivered photosensitiser and elicit the photodynamic response. Light diffusion through newborn calf serum was investigated using the apparatus described by Donnelly et al. [19]. Briefly, silicone wells of defined thicknesses were used to vary the depth of serum through which light from a Paterson Lamp (Phototherapeutics Ltd., Manchester, UK) diffused. The lamp (635 nm, 100 mW cm^{-2}) was set perpendicularly 1.0 cm from a levelled glass plate to which the silicone wells were secured using high vacuum grease. A light detector (Orion PD, Ophir Optronics Inc., Wilmington MA, USA) set to detect at 635 nm was placed 1.0 cm below the glass plate. The influence of increasing concentrations of dissolved photosensitiser on light penetration through a defined thickness of serum was investigated for both TMP and MB. Results were reported as the means (±SD) of five replicates.

2.7. Microbiological investigation-planktonic grown culture

The inoculum of MRSA 180 to be tested was prepared by growing the organism overnight at 37 °C in Mueller–Hinton broth (MHB) and adjusting the turbidity of the overnight culture in MHB to an optical density at 550 nm equivalent to 1×10^8 cfu/ml. Aliquots (100 μl) were then added to the wells of a black-walled 96-well microtitre tray (Nalgene Nunc International, Rochester, NY, USA) and 100 μl of either sterile phosphate buffered saline (PBS) pH 7.4 (control), or sterile PBS containing a defined concentration of MB or TMP (test) were added. Four replicate wells were used for the control and for each MB or TMP concentration tested.

Microtitre trays were incubated for defined time intervals at 37 °C and then either irradiated or subjected to an equivalent dark period. Irradiation (635 nm) was performed using the Paterson Lamp. The fluence rate was 100 mW cm^{-2} and the total light dose delivered was 100 J cm^{-2}. Samples were then removed from each well, serially tenfold diluted in PBS and plated on Mueller–Hinton agar (MHA) plates with the total viable count determined after overnight incubation at 37 °C. In each case, results were expressed as the total viable count for the isolate following treatment with a defined concentration of MB or TMP in comparison with the total viable count for the untreated control.

2.8. Microbiological investigation-biofilm grown culture

PVC discs with a diameter of 4.0 mm were prepared from sheets of cured Plastisol® emulsion and placed in the wells of a black-walled 96-well microtitre tray. An initial inoculum of 1×10^8 cfu/ml was used for all biofilm investigations and this was obtained by diluting an overnight culture in MHB, as described previously. Biofilms were formed by adding 100 μl of the initial inoculum to the wells of the microtitre tray, which was then incubated at 37 °C for 24 h. Following biofilm formation, the PVC discs were removed and gently washed three times with sterile PBS to remove any non-adherent bacteria. The discs were then placed in empty wells of the trays and 100 μl of a MB or TMP solution added. Following incubation with the photosensitiser for a defined time period, the discs were removed from the photosensitiser solution, gently washed in PBS as described previously [18,19] and irradiated (100 J cm^{-2}) in a different well, as described above. Following irradiation, the discs were washed and placed in 2.5 ml PBS in sterile McCartney bottles and bacteria retained on the surface dislodged by mild ultrasonication (5 min) in a 150 W ultrasonic bath operating at a nominal frequency of 50 Hz followed by rapid vortex mixing (30 s). Serial 10-fold dilutions were performed and after overnight incubation, total viable counts were determined with the results expressed relative to the surface area of the PVC disc (cfu/cm^2). In each case, the number of viable bacteria adhering to the PVC disc following treatment with a defined concentration of MB or TMP was compared with the number adhering to the untreated control PVC disc.

2.9. Effect of newborn calf serum on photodynamic killing

Photosensitiser solutions were made up under aseptic conditions in newborn calf serum instead of PBS. Experiments were then simply repeated as above for planktonic and biofilm-grown MRSA 180.

2.10. Statistical analysis

The effects of inclusion of photosensitisers on hardness, compressibility and dermal adhesion were evaluated using a analysis of variance (ANOVA). Post-hoc analysis (Tukey's HSD test) was employed for comparison of the means of the individual groups. In addition, Student's *t*-test was used to evaluate the effect of newborn calf serum on PVA–borate hydrogels and to compare the percentage of each photosensitiser released after 6 h. Microbiological data were analysed, where appropriate, using the Mann–Whitney U test. In all cases, $p < 0.05$ denoted significance.

3. Results

The results in Table 1 show that increasing the photosensitiser concentration up to 1.0 mg ml^{-1} of either MB or TMP had no significant effect on the hardness, compressibility and adhesion of the 8% w/w PVA, 2% w/w borax hydrogel used in this work. Using these three measures, there was no discernable difference between the non-drug-loaded gel and each of the photosensitiser-loaded variants. Also shown in Table 1 is the effect of exposure to calf serum, an addition used to mimic the possible effects of an encounter with excessive wound exudates. There is no evidence of any loss of hardness as a result of application of 20 ml of newborn calf serum. Further detail of the influence of serum on hardness, with respect to the time of exposure, is shown in Fig. 2. It is clear that serum has

Table 1
Influence of drug loadings of 1.0 mg ml^{-1} and 360 min of exposure to 20 ml newborn calf serum* on the physical properties of 8.0% w/w PVA/ 2.0% w/w borax hydrogels. Means ± SD, n = 4. Statistical analysis was by a one-way ANOVA.

Property	1 mg ml^{-1} TMP	No drug	P value
Adhesion (N cm^2)	1.97 ± 0.241	2.09 ± 0.132	0.678
Compressibility (N s)	5.97 ± 0.513	6.67 ± 0.194	0.101
Hardness (N)	6.19 ± 0.528	6.81 ± 0.151	0.186
Hardness (serum affect)* (N)	6.08 ± 0.500	6.86 ± 0.781	0.216
	1 mg ml^{-1} MB	No drug	P value
Adhesion (N cm^2)	2.09 ± 0.149	2.09 ± 0.132	0.987
Compressibility (N s)	6.52 ± 0.496	6.67 ± 0.194	0.878
Hardness (N)	6.76 ± 0.549	6.81 ± 0.151	0.988
Hardness (serum affect)* (N)	6.38 ± 0.471	6.86 ± 0.781	0.519

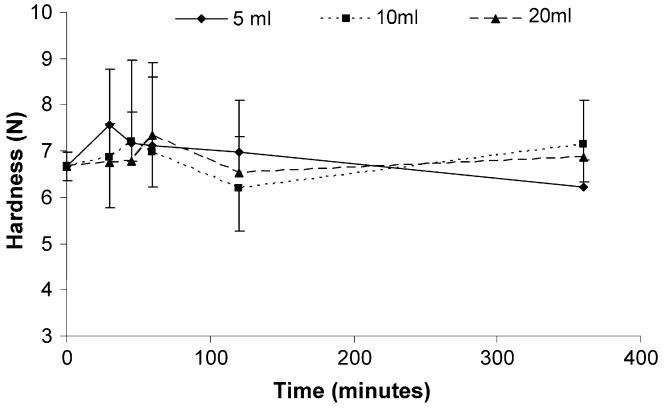

Fig. 2. Influence of volume of newborn calf serum and time on hardness of a PVA–borate hydrogel. The PVA–borate hydrogel used was that containing 8% w/w PVA and 2.0% w/w borax. Means ± SD, n = 4.

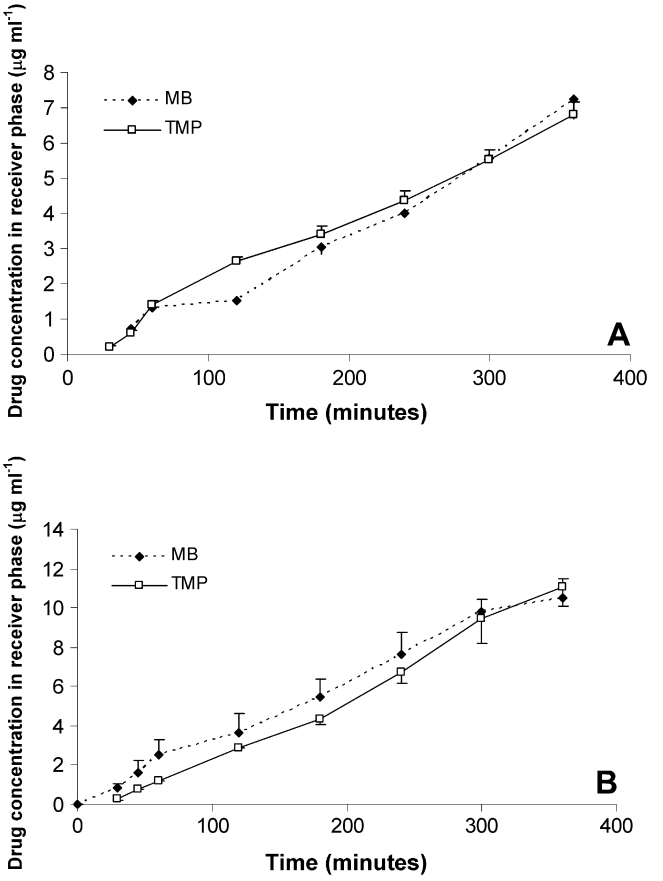

Fig. 3. Influence of temperature on release of photosensitisers from a PVA–borate hydrogel containing 8% w/w PVA and 2.0% w/w borax. The drug loading in each case was 1 mg ml^{-1}. The temperatures employed were 25 °C (A) and 37 °C (B). Means ± SD, n = 3.

only a limited effect on the hardness of the hydrogel, with none of the volumes investigated having a significant effect on the physical properties of the hydrogel, even after 6 h. When taken together, these findings indicate that the gel is resistant to excessive moisture and can accommodate a limited amount of payload. Increasing MB loading beyond 1.0 mg ml^{-1} led to formation of drug aggregates, which precipitated out of solution as solid particles surrounded by gel. This affected the physical properties of the hydrogel adversely, such that reproducible measurements of physical properties were not possible. This effect was not observed with TMP. However, to allow reasonable comparisons to be made, the drug loading was not increased further for either drug.

The in vitro release profiles for MB and TMP from the 8% w/w PVA, 2.0% w/w borax hydrogel are shown in Fig. 3. The release profiles for both MB and TMP are comparable with no significant difference found in the mass of photosensitiser released after 6 h at each temperature. At 25 °C (Fig. 3A and after 6 h, the concentration in the receiver phase was approximately 7 μg ml^{-1} for both drugs, which equates to 16.08% of the total MB loading and 17.05% of the total TMP loading in the gel sample. Increasing the temperature to 37 °C (Fig. 3B) significantly increased drug release rate, with approximately 11 μg ml^{-1} for both drugs found in the receiver phase and again equating to 26.27% and 27.28% of MB and TMP loading, respectively, released from the formulation.

The data in Fig. 4 gives an indication of the effect of serum thickness and the presence of photosensitiser on the light fluence. In Fig. 4A, it can be seen that as serum depth increased, the measured light fluence at 635 nm decreased in an almost linear fashion. However, the expected decrease amounted to approximately 15%. The effect of photosensitiser in the serum layer was more prominent. Fig. 4B shows that, for a given serum depth (0.75 mm), increasing the MB concentration caused a marked decrease in the measured fluence. This decline in measured fluence, though still significant, was not as strongly observed with the presence of TMP. Serum containing MB reduced the measured light fluence to a much greater extent than that containing TMP at all concentrations apart from 5.0 mg ml^{-1} and the difference in measured fluence here was still significant ($p < 0.0001$).

Tables 2–5 show the results of microbiological experiments carried out in this study. Incubation of the MRSA isolate with either TMP or MB caused a decrease in the number of viable cells, even in the absence of irradiation (Table 2). The magnitude of this toxic effect increased significantly with increasing concentration. The mean percentage kill was increased significantly by irradiation in the majority of cases (Table 2). With MB, the type of culture grown had a significant effect on the efficacy of photodynamic inactivation. For example, when the isolate was grown planktonically and irradiated at a light dose of 100 J cm^{-2} after incubation with 10 μg ml^{-1} MB, a mean percentage kill of >99.99% was achieved. In contrast, the mean percentage kill for the isolate grown in biofilm culture for the same drug concentration and light dose was significantly less at 88.19% ($p = 0.0209$). This effect was not observed for TMP, where the mean percentage kill of planktonic and biofilm cultures were similar. For example, the mean percentage kill for a planktonic culture exposed to 10 μg ml^{-1} TMP and a light dose of 100 J cm^{-2} was >99.99% and the mean percentage kill of the equivalent biofilm culture was 99.71% ($p = 0.0833$).

When the mean percentage kill was less than 90%, the experiment was repeated using a light dose of 200 J cm^{-2}. In the majority

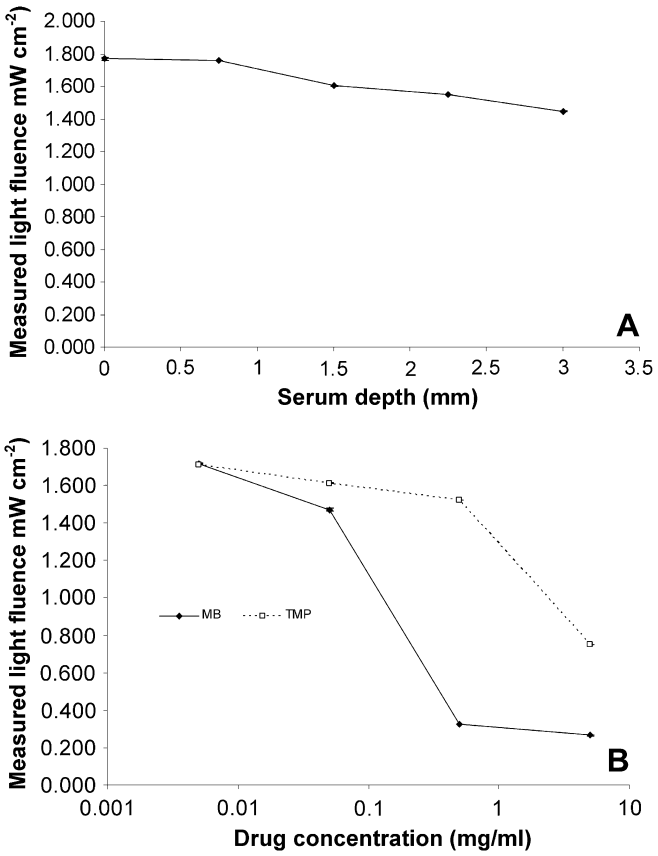

Fig. 4. Influence of serum depth (A) and drug concentration (B) on permeation of light (635 nm, 100 mw cm^{-2}) from a Paterson lamp. Means ± SD, $n = 5$.

of cases, this resulted in a significant increase in the mean percentage kill (Table 3). For example, in the case of a planktonic culture incubated in the presence of 2.0 μg ml^{-1} TMP, and irradiated at a light dose of 100 J cm^2, the associated mean percentage kill was 39.64%. When the light dose was doubled, the mean percentage kill increased significantly to >99.99% ($p = 0.0209$). For all cultures irradiated with a light dose of 200 J cm^2 in the presence of TMP, the mean percentage kill achieved was >94.92% in each case. However, even this increase in light dose led to unacceptable kill rates for MB concentrations of 2.0, 5.0, and 10 μg ml^{-1} (Table 3).

It was notable that, for TMP, the presence of calf serum had no significant effect on the mean percentage kill (Table 4). For example, the mean percentage kill for the planktonic and biofilm cultures incubated with 10 μg ml^{-1} TMP in the presence of calf serum and a light dose of 100 J cm^{-2} were 99.95% ($p > 0.9999$) and 99.66% ($p > 0.9999$), respectively. Neither of these kill rates were significantly different from the mean percentage kills achieved when the isolate was incubated with the equivalent TMP concentrations in PBS pH 7.4. With MB, kill rates were generally significantly reduced by the presence of calf serum (Table 4). For example, planktonically grown MRSA incubated with 50 μg ml^{-1} MB in PBS pH 7.4 yielded a mean percentage kill of >99.99% upon irradiation, whereas 50 μg ml^{-1} MB in calf serum gave a significantly lower mean percentage kill of 23.08% ($p = 0.0209$). Increasing the light dose to 200 J cm^{-2} had no significant effect on rates of kill in the presence of calf serum, regardless of mode of growth or which photosensitiser was used (Table 5).

4. Discussion

The human body, especially the skin surface, is not a sterile environment, even in the absence of infection [23]. Therefore, when no infection is present, equilibrium exists between host

Table 2
Effect of a 30 min incubation with MB and TMP solutions followed by irradiation with a Paterson lamp (635 nm, 100 J cm^{-2}) on the viability of an MRSA isolate (MRSA 180) grown planktonically and in biofilms. S−L−: incubation with PBS pH 7.4 and then subjected to an equivalent dark period. S−L+: incubation with PBS pH 7.4 and irradiated. S+L+: incubation with photosensitiser and then irradiated. S+L−: incubation with photosensitiser and then subjected to an equivalent dark period. Means ± SD, $n = 4$.

Drug concentration (μg ml^{-1})	Light treatment	MB		TMP	
		Surviving organisms (±SD) $n = 5$	Mean % Kill	Surviving organisms (±SD) $n = 5$	Mean % Kill
Planktonic		(CFU ml^{-1})		(CFU ml^{-1})	
0.0	S−L−	5.50 (±0.41) × 10^7	0.00	3.50 (±4.76) × 10^7	0.00
0.0	S−L+	6.15 (±3.47) × 10^7	0.00	3.35 (±1.28) × 10^7	4.29
2.0	S+L−	3.90 (±0.48) × 10^7	29.73	3.25 (±0.72) × 10^7	7.14
2.0	S+L+	4.60 (±1.49) × 10^6	91.71	3.35 (±1.09) × 10^7	4.29
5.0	S+L−	2.75 (±0.25) × 10^7	50.45	3.50 (±0.77) × 10^7	0.00
5.0	S+L+	7.00 (±9.34) × 10^5	98.74	5.83 (±6.31) × 10^6	83.36
10.0	S+L−	4.25 (±0.47) × 10^7	23.42	1.95 (±0.48) × 10^7	44.43
10.0	S+L+	4.16 (±3.57) × 10^2	>99.99	1.69 (±2.18) × 10^3	>99.99
50.0	S+L−	5.15 (±0.94) × 10^5	99.07	1.86 (±0.15) × 10^7	47.00
50.0	S+L+	0.00 (±0.00)	100.00	0.00 (±0.00)	100.00
250.0	S+L−	2.97 (±1.71) × 10^5	99.46	3.85 (±0.41) × 10^7	0.00
250.0	S+L+	0.00 (±0.00)	100.00	0.00 (±0.00)	100.00
Biofilm		(CFU cm^{-2})		(CFU cm^{-2})	
0.0	S−L−	1.44 (±1.44) × 10^7	0.00	1.40 (±0.87) × 10^7	0.00
0.0	S−L+	5.00 (±2.30) × 10^7	0.00	1.20 (±1.17) × 10^7	14.29
2.0	S+L−	1.98 (±0.83) × 10^7	0.00	9.50 (±8.69) × 10^6	32.14
2.0	S+L+	1.70 (±0.12) × 10^7	0.00	1.19 (±1.18) × 10^6	91.52
5.0	S+L−	3.14 (±2.54) × 10^7	0.00	4.63 (±0.63) × 10^6	66.96
5.0	S+L+	2.04 (±2.60) × 10^6	85.85	1.26 (±0.10) × 10^6	99.10
10.0	S+L−	4.11 (±4.13) × 10^7	0.00	1.73 (±1.07) × 10^6	87.68
10.0	S+L+	1.70 (±1.21) × 10^6	88.19	4.00 (±5.15) × 10^4	99.71
50.0	S+L−	9.58 (±1.12) × 10^6	33.51	1.5 (±0.94) × 10^5	98.93
50.0	S+L+	7.92 (±6.07) × 10^3	99.95	6.25 (±7.50) × 10^2	>99.99
250.0	S+L−	6.25 (±2.30) × 10^6	56.60	1.64 (±0.74) × 10^4	99.88
250.0	S + L+	2.06 (±1.39) × 10^3	99.99	0.00 (±0.00)	100.00

Table 3
Effect of increasing the light dose to 200 J cm^{-2} on level of TMP- and MB-mediated photosensitisation. All photosensitisers prepared in sterile PBS pH 7.4. S = sensitiser, L = light (as before). N.S. = not sampled, as percentage kill >90% with light dose of 100 J cm^{-2}. Means ± SD, $n = 4$.

Drug concentration (μg ml^{-1})	Light treatment	MB		TMP	
		Surviving organisms (±SD) $n = 5$	Mean % kill	Surviving organisms (±SD) $n = 5$	Mean % kill
Planktonic		(CFU ml^{-1})		(CFU ml^{-1})	
0.0	S−L−	$3.50\ (\pm0.48) \times 10^7$	0.00	$4.30\ (\pm1.05) \times 10^7$	0.00
0.0	S−L+	$3.35\ (\pm1.28) \times 10^7$	4.29	$4.25\ (\pm0.77) \times 10^7$	29.17
2.0	S+L−	$3.10\ (\pm1.16) \times 10^7$	11.43	$5.20\ (\pm0.59) \times 10^7$	13.33
2.0	S+L+	$4.10\ (\pm4.78) \times 10^4$	99.88	$2.15\ (\pm1.25) \times 10^3$	>99.99
5.0	S+L−	$3.00\ (\pm0.43) \times 10^7$	14.29	$3.75\ (\pm1.02) \times 10^7$	37.50
5.0	S+L+	$5.80\ (\pm10.00) \times 10^2$	>99.99	$8.43\ (\pm9.56) \times 10^2$	>99.99
10.0	S+L−	N.S.	–	N.S.	–
10.0	S+L+	N.S.	–	N.S.	–
50.0	S+L−	N.S.	–	N.S.	–
50.0	S+L+	N.S.	–	N.S.	–
250.0	S+L−	N.S.	–	N.S.	–
250.0	S+L+	N.S.	–	N.S.	–
Biofilm		(CFU cm^{-2})		(CFU cm^{-2})	
0.0	S−L−	$2.21\ (\pm1.75) \times 10^7$	0.00	$2.21\ (\pm1.75) \times 10^7$	0.00
0.0	S−L+	$2.49\ (\pm1.94) \times 10^7$	0.00	$2.49\ (\pm1.95) \times 10^7$	0.00
2.0	S + L−	$8.63\ (\pm4.44) \times 10^6$	60.97	$1.80\ (\pm1.00) \times 10^7$	18.55
2.0	S+L+	$1.03\ (\pm0.81) \times 10^7$	53.62	$2.56\ (\pm3.17) \times 10^5$	98.84
5.0	S+L−	$1.90\ (\pm0.89) \times 10^7$	14.02	N.S.	–
5.0	S+L+	$1.24\ (\pm0.62) \times 10^7$	44.00	N.S.	–
10.0	S+L−	$1.88\ (\pm0.76) \times 10^7$	15.16	N.S.	–
10.0	S+L+	$1.28\ (\pm1.59) \times 10^7$	42.14	N.S.	–
50.0	S+L−	N.S.	–	N.S.	–
50.0	S+L+	N.S.	–	N.S	–
250.0	S+L−	N.S.	–	N.S	–
250.0	S+L+	N.S.	–	N.S	–

Table 4
Effect of the presence of calf serum on MB- and TMP-mediated lethal photosensitisation of an MRSA isolate (MRSA 180) grown planktonically and in biofilms. S−L−: incubated with calf serum and then subjected to an equivalent dark period. S−L+: incubation with calf serum and irradiated. S+L+: incubation with photosensitiser in calf serum and then irradiated. S+L−: incubation with photosensitiser in calf serum and then subjected to an equivalent dark period. Means ± SD, $n = 4$.

Drug concentration (μg ml^{-1})	Light treatment	MB		TMP	
		Surviving organisms (±SD) $n = 5$	Mean % kill	Surviving organisms (±SD) $n = 5$	Mean % kill
Planktonic		(CFU ml^{-1})		(CFU ml^{-1})	
0.0	S−L−	$3.90\ (\pm0.74) \times 10^7$	0.00	$4.40\ (\pm0.43) \times 10^7$	0.00
0.0	S−L+	$4.75\ (\pm1.35) \times 10^7$	0.00	$4.15\ (\pm1.20) \times 10^7$	5.68
2.0	S+L−	$5.05\ (\pm1.30) \times 10^7$	0.00	$4.00\ (\pm1.47) \times 10^7$	9.09
2.0	S+L+	$4.75\ (\pm2.18) \times 10^7$	0.00	$2.96\ (\pm4.19) \times 10^5$	99.33
5.0	S+L−	$4.15\ (\pm1.14) \times 10^7$	0.00	$4.15\ (\pm0.89) \times 10^7$	5.68
5.0	S+L+	$5.00\ (\pm1.77) \times 10^7$	0.00	$3.90\ (\pm0.99) \times 10^4$	99.91
10.0	S+L−	$5.55\ (\pm1.61) \times 10^7$	0.00	$4.70\ (\pm0.99) \times 10^6$	0.00
10.0	S+L+	$4.30\ (\pm1.09) \times 10^7$	0.00	$2.21\ (\pm1.01) \times 10^4$	99.95
50.0	S+L−	$6.60\ (\pm5.60) \times 10^7$	0.00	$6.40\ (\pm2.59) \times 10^6$	85.45
50.0	S+L+	$3.00\ (\pm2.05) \times 10^7$	23.08	$5.46\ (\pm7.76) \times 10^3$	99.99
250.0	S+L−	$1.18\ (\pm0.41) \times 10^7$	69.87	$7.10\ (\pm4.48) \times 10^4$	99.83
250.0	S+L+	$1.36\ (\pm0.30) \times 10^5$	99.65	$7.53\ (\pm9.04) \times 10^2$	>99.99
Biofilm		(CFU cm^{-2})		(CFU cm^{-2})	
0.0	S−L−	$2.26\ (\pm0.40) \times 10^7$	0.00	$1.54\ (\pm0.41) \times 10^8$	0.00
0.0	S−L+	$3.01\ (\pm1.42) \times 10^7$	0.00	$8.00\ (\pm0.91) \times 10^6$	94.81
2.0	S+L−	$4.16\ (\pm2.88) \times 10^7$	0.00	$2.70\ (\pm3.30) \times 10^7$	82.47
2.0	S+L+	$4.31\ (\pm3.05) \times 10^7$	0.00	$2.55\ (\pm2.06) \times 10^7$	83.44
5.0	S+L−	$3.86\ (\pm1.75) \times 10^7$	0.00	$2.03\ (\pm1.34) \times 10^7$	86.85
5.0	S+L+	$1.55\ (\pm0.97) \times 10^7$	31.42	$1.40\ (\pm1.49) \times 10^7$	90.91
10.0	S+L−	$2.98\ (\pm1.99) \times 10^7$	0.00	$9.25\ (\pm14.87) \times 10^6$	93.99
10.0	S+L+	$1.80\ (\pm2.17) \times 10^7$	20.35	$5.25\ (\pm1.55) \times 10^5$	99.66
50.0	S+L−	$3.80\ (\pm2.23) \times 10^7$	0.00	$2.46\ (\pm1.58) \times 10^7$	84.01
50.0	S+L+	$2.80\ (\pm2.12) \times 10^7$	0.00	$6.00\ (\pm2.89) \times 10^5$	99.61
250.0	S+L−	$3.46\ (\pm2.22) \times 10^7$	0.00	$1.58\ (\pm1.12) \times 10^7$	89.77
250.0	S+L+	$1.13\ (\pm0.42) \times 10^6$	94.99	$6.09\ (\pm5.46) \times 10^4$	99.96

resistance to infection and the virulence of attacking microorganisms. Eventually, host immunity overcomes the invaders and removes any infecting microorganisms [24]. However, violation of intact skin, whether as a result of surgery, burning or trauma, causes a change in this equilibrium. Impaired host defences, increased levels of bacteria and several other mechanisms can cause a clinical infection to result. The majority of wound infections are bacterial in origin and these infections are difficult to eradicate, being caused in many cases by antibiotic-resistant bacteria such as MRSA.

Table 5
Effect of increasing the light dose to 200 J cm^{-2} on level of TMP- and MB-mediated lethal photosensitisation of an MRSA isolate (MRSA 180) growing planktonically and in biofilm. All photosensitisers prepared in newborn calf serum. S = sensitiser, L = light (as before). N.S. = not sampled, as percentage kill >90% with light dose of 100 J cm^{-2}. Means ± SD, $n = 4$.

Drug concentration ($\mu g\ ml^{-1}$)	Light treatment	MB		TMP	
		Surviving organisms (±SD) $n = 5$	Mean % kill	Surviving organisms (±SD) $n = 5$	Mean % kill
Planktonic		($CFU\ ml^{-1}$)		($CFU\ ml^{-1}$)	
0.0	S−L−	$6.00\ (\pm1.17) \times 10^7$	0.00	N.S.	–
0.0	S−L+	$5.60\ (\pm0.97) \times 10^7$	6.67	N.S.	–
2.0	S+L−	$5.40\ (\pm2.47) \times 10^7$	10.00	N.S.	–
2.0	S+L+	$5.25\ (\pm1.39) \times 10^7$	12.50	N.S.	–
5.0	S+L−	$5.40\ (\pm1.14) \times 10^7$	10.00	N.S.	–
5.0	S+L+	$4.80\ (\pm0.83) \times 10^7$	20.00	N.S.	–
10.0	S+L−	$3.65\ (\pm0.96) \times 10^7$	39.17	N.S.	–
10.0	S+L+	$4.20\ (\pm0.90) \times 10^7$	30.00	N.S.	–
50.0	S+L−	$5.45\ (\pm0.90) \times 10^7$	9.17	N.S.	–
50.0	S+L+	$1.49\ (\pm0.02) \times 10^7$	75.17	N.S.	–
250.0	S+L−	N.S.	–	N.S.	–
250.0	S+L+	N.S.	–	N.S.	–
Biofilm		($CFU\ cm^{-2}$)		($CFU\ cm^{-2}$)	
0.0	S−L−	$2.14\ (\pm0.30) \times 10^7$	0.00	$6.88\ (\pm1.25) \times 10^7$	0.00
0.0	S−L+	$2.81\ (\pm1.55) \times 10^7$	0.00	$1.28\ (\pm0.48) \times 10^8$	0.00
2.0	S+L−	$4.45\ (\pm3.78) \times 10^7$	0.00	$1.10\ (\pm0.58) \times 10^8$	0.00
2.0	S+L+	$3.35\ (\pm1.95) \times 10^7$	0.00	$7.83\ (\pm12.14) \times 10^6$	88.62
5.0	S+L−	$2.65\ (\pm0.40) \times 10^7$	0.00	$2.03\ (\pm1.84) \times 10^7$	70.53
5.0	S+L+	$1.63\ (\pm0.70) \times 10^7$	24.07	$7.88\ (\pm5.91) \times 10^4$	99.89
10.0	S+L−	$1.11\ (\pm19.49) \times 10^6$	94.81	N.S.	–
10.0	S+L+	$1.86\ (\pm0.63) \times 10^7$	13.26	N.S.	–
50.0	S+L−	$1.02\ (\pm2.54) \times 10^7$	52.39	N.S.	–
50.0	S+L+	$1.70\ (\pm0.41) \times 10^7$	20.56	N.S.	–
250.0	S+L−	$2.17\ (\pm1.85) \times 10^7$	0.00	N.S.	–
250.0	S+L+	$1.08\ (\pm0.95) \times 10^6$	94.97	N.S	–

Alternative antimicrobial methods for the treatment of wound infections, which would eradicate infecting bacteria without the development of resistance, would be extremely beneficial. PACT using photosensitisers of different chemical classes, such as phenothiaziniums [7], porphyrins [9] and phthalocyanines [8] has been shown to kill effectively MRSA strains growing planktonically with no reports on the development of resistance to PACT by MRSA currently existing. As cationic photosensitisers are well known to be more effective than their anionic counterparts in PACT of Gram-positive as well as Gram-negative bacteria [5,6,25], we used two cationic photosensitisers from different chemical classes, MB and TMP, in the present study to investigate the potential utility of PACT in the treatment of wound infections.

If PACT is to be employed successfully for the treatment of wound infections, the photosensitiser and light doses employed must be capable of delivery to the wound. Light delivery to the skin is simple and has been frequently employed in photodynamic therapy (PDT) of several different dysplastic and neoplastic diseases [3]. In PDT, however, the photosensitiser or its precursor is typically administered orally or intravenously. Due to disordered metabolism and blood flow peculiar to dysplastic or neoplastic tissue, photosensitising concentrations of drug accumulate in the target lesions relatively quickly. Targeting of photosensitisers to wound infections in this way is not possible and, therefore, the drug must be applied topically. Clearly powder- or solution-based drug delivery systems are not appropriate for photosensitiser administration to wounds, due to problems with accurate dosing and difficulty in retention, particularly in heavily exuding wounds. Consequently, this paper describes the use of a PVA–borate hydrogel as a delivery platform suitable for use in PACT of wound infections. Such a system should ideally be able to be poured into a wound and flow so as to conform to the shape and contours of a wound, thus allowing efficient drug delivery to the entire wound. However, the system should maintain its structural integrity while in place and not be affected in a detrimental manner by absorption of wound exudates. It should release its drug payload efficiently in a reasonable time frame so as to avoid prolonged application. Importantly, such a system should have sufficient hardness to maintain integrity, thereby ensuring trauma-free removal from the wound prior to irradiation. This is because "self-shielding" of light, due to absorption by photosensitiser still within the device, may prevent efficient light delivery to photosensitiser molecules within, or in close proximity to, microbial cells [26]. This latter effect could severely reduce the success of the treatment.

Following detailed analysis of physical characterisation results, the 8% w/w PVA/ 2.0% w/w borax hydrogel was chosen as the formulation possessing properties most suited to the chosen application. This formulation was then used for all further investigations.

PVA–borate hydrogels possess unique flow properties. Borate ions, produced through the dissociation of sodium tetraborate decahydrate (borax) to equi-molar portions of boric acid, borate ions and sodium ions, form a thermo-reversible complex with the hydroxyl groups on PVA molecules [27]. The work of Loughlin et al. [16] has highlighted how the unique flow properties of drug-loaded PVA–borate hydrogels have potential as biomedical hydrogels for the treatment of ulcerated and lacerated skin lesions. They possess the ability to flow into, and produce intimate contact with, ulcerated wounds, whilst their dilatant structure allows for intact removal once treatment is complete. Moreover, their high water content (>90% water) offers the ability to produce a beneficial moist environment for the enhancement of autolytic wound debridement [28].

In the present study, an 8.0% w/w PVA, 2.0% w/w borax hydrogel was selected as the formulation possessing properties most suitable for a PACT delivery system for wound infections. This choice was based on preliminary evaluations, giving an optimised formulation that was used further to investigate the effect of newborn calf serum on the physical integrity of the gel, which was represented by measurements of hardness. It was found that newborn calf serum had no significant effect on gel hardness over the entire

time period of the study (6 h), regardless of the volume of serum used. Therefore, it may be reasonably assumed that insertion of the hydrogel into a moderately exuding wound will leave the formulation intact for the duration of application. This will then allow complete removal of the formulation, hence, avoiding interference with irradiation. Furthermore, incorporating photosensitisers in concentrations up to of 1.0 mg ml^{-1} into the optimised hydrogel had no significant effect on its physical properties, regardless of whether serum was present or not.

Following initial lag periods, photosensitiser release from the PVA–borate hydrogel followed approximately zero-order kinetics in each case. However, drug release form the PVA–borate hydrogel was low. This may partly be explained by the inherent viscosity of these hydrogels and low drug loading, leading to a reduced concentration gradient driving diffusion. Furthermore, as there was no difference in release of the two compounds despite differences in their molecular size, it is also likely that electrostatic binding of the photosensitiser molecules by the PVA–borate polyelectrolyte complex had an influence on drug release. MB and TMP are both cationic compounds, as shown in Fig. 1, and such compounds have been shown to interact strongly with negatively charged poly(electrolytes) [29]. Previous studies have shown a similar reduction in drug release as a result of electrostatic interaction between poly(electrolytes) and both MB and other cationic drugs [30,31]. However, the poly(electrolyte) complex in PVA–borate hydrogels is sensitive to heat and by increasing the temperature to 37 °C, greater drug release was attained (Fig. 3). This may be due to enhanced borate dissociation from the PVA–borate network or by enhanced diffusivity through the gel matrix.

Despite the relatively low percentages of photosensitisers released, the receiver phase concentrations of both drugs achieved after 6 h release exceeded the concentrations required for high rates (>95%) of kill of an MRSA isolate grown planktonically and in biofilm. Furthermore, the receiver phase volume used in this study was set at 100 ml to ensure preservation of sink conditions and fluid volumes in wounds are unlikely to be this high. Therefore, the in vivo photosensitiser concentrations achievable would be considerably greater and it could be argued that higher rates of kill would ensue. However, further incubation studies would be required to confirm this assertion.

The concentrations of TMP and MB required to induce high kill rates upon irradiation in this study were approximately 1000-fold lower than those of Toluidine Blue O (TBO) (a phenothiazinium compound closely related to MB) required to induce similar kill rates in *Candida albicans* [18], and those of TBO and TMP required to induce similar kill rates in *Pseudomonas aeruginosa* [19]. This is likely to be due to the known greater susceptibility of Gram-positive bacteria, such as *S. aureus,* to the toxic effects of PACT as compared to fungi and Gram-negative organisms [5,6].

Wounds, particularly those which are heavily exuding, are likely to contain a significant amount of fluid and an array of cellular products of tissue breakdown as well as immunochemicals and inflammatory mediators. Such substances could potentially absorb incident light, reducing its penetration and also interact in a number of ways with photosensitiser molecules, reducing their effectiveness. It has been previously shown that serum, used to simulate the conditions in a wound, can significantly reduce the effectiveness of PACT [7]. In the present study, TMP-mediated photodynamic killing was not affected by the presence of calf serum which was used to simulate the presence of extracellular material in the wound. This was despite the fact that serum was capable of significantly reducing the fluence of incident light and that this was further reduced when the serum contained photosensitisers. However, MB-mediated photodynamic killing was adversely affected. This may be due to binding of MB by extracellular material, which could prevent uptake. These findings are in agreement with those of Nitzan et al. [32], who postulated that proteins in serum may interact with photosensitisers and render them unavailable for penetration through bacterial membranes. The exact composition of the extracellular material, with respect to hydrophobicity and charge, may cause the selective binding of MB over TMP. [33].

The Paterson lamp (Xenon/Ceramic/300 watts, filtered output: 635 ± 25 nm, 100 mW cm^{-2}) was used as the light source in this study. With respect to photodynamic treatments, this is a widely available source in the UK, is simple to operate and was already in use in our laboratory. Lasers tuned to a specific photosensitiser wavelength are not practical for routine use as they can, by definition, only be used with one photosensitiser, are expensive and require specialist assistance with operation and maintenance. TMP, a porphyrin, has an absorption maximum at 421.6 nm with only a minor absorption band at 639 nm, such that its absorptivity at 639 nm is only 6% of that at 421.6 nm [34]. Consequently, the wavelength used in the present study for irradiating TMP, 635 nm, may lead to reduced singlet oxygen production due to inefficient photosensitiser excitation. MB, being a phenothiazinium compound, will absorb light of 635 nm, as delivered by the Paterson lamp, strongly. However, we found TMP to be considerably more effective than MB in this study. It is possible that this is due to its enhanced cationic character relative to MB, meaning it is more efficiently absorbed by, or bound to, MRSA 180 [5,6]. Our results also demonstrate that a single light source can be effectively utilised for irradiation of different photosensitisers, thereby reducing costs if the system was used in clinical practice.

In general, the critical number of bacteria required to cause wound infection has been shown to be about 10^5 bacteria per gram of tissue. With less than this number, there is a 94% chance of successful wound closure. However, if more than 10^5 bacteria are present, the chance of successful wound closure is significantly lowered [23,24,35,36]. It is notable that in this study, despite achieving rates of kill of >95% upon irradiation, $>10^5$ bacteria remained in many cases. This is, at least in part, due to the high bacterial challenges used. However, these may actually be reflective of the clinical situation in the case of a severe wound infection. PACT is not associated with resistance, even upon multiple treatments [5,6]. In addition, the photosensitisers employed in this technique exhibit selectivity for uptake into microbial cells compared to their human counterparts and, hence, no damage to the host should result during treatment [36]. Therefore, PACT could safely be repeated on many occasions with a view to reducing the bacterial load below 10^5 per gram of tissue. The system described here should allow such routine repeat applications and removals and it could be easily used in both primary and secondary care by trained healthcare professionals. As a result, it has the potential to improve the treatment of infected wounds significantly, thereby decreasing costs for healthcare providers.

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References

[1] R.S. Howell-Jones, M.J. Wilson, K.E. Hill, P.E. Price, D.W. Thomas, A review of the microbiology, antibiotic usage and resistance in chronic skin wounds, J. Antimicrob. Chemother. 55 (2005) 143–149.
[2] M. Michel, L. Gutmann, Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*: therapeutic realities and possibilities, Lancet 349 (1997) 1901–1906.

[3] R.F. Donnelly, P.A. McCarron, A.D. Woolfson, Drug delivery of aminolevulinic acid from topical formulations intended for photodynamic therapy, Photochem. Photobiol. 81 (2005) 750–767.
[4] J. Moan, Q. Peng, An outline of the hundred-year history of PDT, Anticancer Res. 23 (2003) 3591–3600.
[5] G. Jori, S.B. Brown, Photosensitized inactivation of microorganisms, Photochem. Photobiol. Sci. 3 (2004) 403–405.
[6] M. Wainwright, K.B. Crossley, Photosensitising agents – circumventing resistance and breaking down biofilms: A review, Int. Biodeter. Biodegr. 53 (2004) 119–126.
[7] M. Wilson, J. Pratten, Lethal photosensitisation of *Staphylococcus aureus* in vitro: effect of growth phase, serum and pre-irradiation time, Laser. Surg. Med. 16 (1995) 272–276.
[8] M.A. Griffiths, B.W. Wren, M. Wilson, Killing of methicillin-resistant Staphylococcus aureus in vitro using aluminium disulphonated phthalocyanine, a light-activated antimicrobial agent, J. Antimicrob. Chemother. 40 (1997) 873–876.
[9] S. Ferro, F. Ricchelli, G. Mancini, G. Tognon, G. Jori, Inactivation of methicillin-resistant *Staphylococcus aureus* (MRSA) by liposome-delivered photosensitising agents, J. Photochem. Photobiol. B 83 (2006) 98–104.
[10] R.R.A. Hayek, N.S. Araujo, M.A. Gioso, J. Ferreira, C.A. Baptista-Sobrinho, A.M. Yamada, M.S. Ribeiro, Comparative study between the effects of photodynamic therapy and conventional therapy on microbial reduction in ligature-induced peri-implantitis in dogs, J. Periodontol. 76 (2005) 1275–1281.
[11] B.W. Sigusch, A. Pfitzner, V. Albrecht, E. Glockmann, Efficacy of photodynamic therapy on inflammatory signs and two selected periodontopathogenic species in a beagle dog model, J. Periodontol. 76 (2005) 1100–1105.
[12] M.C. Teichert, J.W. Jones, M.N. Usacheva, M.A. Biel, Treatment of oral candidiasis with Methylene Blue-mediated photodynamic therapy in an immunodeficient murine model, Oral Surg. Oral Med. O 93 (2002) 155–160.
[13] F. Gad, T. Zahra, K.P. Francis, T. Hasan, M.R. Hamblin, Targeted photodynamic therapy of established soft-tissue infections in mice, Photochem. Photobiol. Sci. 3 (2004) 451–458.
[14] S.A. G Lambrechts, T.N. Demidova, M.C.G. Aalders, T. Hasan, M.R. Hamblin, Photodynamic therapy for *Staphylococcus aureus* infected burn wounds in mice, Photochem. Photobiol. Sci. 4 (2005) 503–509.
[15] P.A. McCarron, R.F. Donnelly, A. Zawislak, A.D. Woolfson, J.H. Price, R. McClelland, Evaluation of a water-soluble bioadhesive patch for photodynamic therapy of vulval lesions, Int. J. Pharm. 293 (2005) 11–23.
[16] R.G. Loughlin, M.M. Tunney, R.F. Donnelly, D.J. Murphy, M.G. Jenkins, P.A. McCarron, Modulation of gel formation and drug release characteristics of lidocaine-loaded, poly(vinyl alcohol)–tetraborate, hydrogel systems using scavenger polyol sugars, Eur. J. Pharm. Biopharm. 69 (2008) 1135–1146.
[17] R.F. Donnelly, P.A. McCarron, J.M. Lightowler, A.D. Woolfson, Bioadhesive patch-based delivery of 5-aminolevulinic acid to the nail for photodynamic therapy of onychomycosis, J. Control. Release 103 (2005) 381–392.
[18] R.F. Donnelly, P.A. McCarron, M.M. Tunney, A.D. Woolfson, Potential of photodynamic therapy in the treatment of fungal infections of the mouth. Design and physicochemical characterisation of a mucoadhesive patch containing Toluidine Blue O, J. Photochem. Photobiol. B 86 (2007) 59–69.
[19] R.F. Donnelly, P.A. McCarron, C.M. Cassidy, J.S. Elborn, M.M. Tunney, Delivery of photosensitisers and light through mucus: investigations into the potential use of photodynamic therapy for treatment of *Pseudomonas aeruginosa* cystic fibrosis pulmonary infection, J. Control. Release 117 (2007) 217–226.
[20] D.S. Jones, A.D. Woolfson, J. Djokic, Texture profile analysis of bioadhesive polymeric semisolids: mechanical characterization and investigation of interactions between formulation components, J. Appl. Polym. Sci. 61 (1996) 2229–2234.
[21] D.S. Jones, A.D. Woolfson, A.F. Brown, W.A. Coulter, C. McClelland, C.R. Irwin, Design, characterisation and preliminary clinical evaluation of a novel mucoadhesive topical formulation containing tetracycline for the treatment of periodontal disease, J. Control. Release 67 (2000) 357–368.
[22] M.A. Punnia, Evaluation of pH changes in inflammation of the subcutaneous air pouch lining in the rat, induced by carrageenan, dextran and staphylococcus, J. Oral Pathol. Med. 16 (1987) 36–44.
[23] W.K. Stadelmann, A.G. Digenis, G.R. Tobin, Impediments to wound healing, Am. J. Surg. 176 (1998) 39S–47S.
[24] M.C. Robson, Wound infection. A failure of wound healing caused by an imbalance of bacteria, Surg. Clin. N. Am. 77 (1997) 637–650.
[25] A. Minnock, D.I. Vernon, J. Schofield, J. Griffiths, J.H. Parish, S.B. Brown, Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria, J. Photochem. Photobiol. B 32 (1996) 159–164.
[26] M.J. Gannon, S.B. Brown, Photodynamic therapy and it's applications in gynaecology, Brit. J. Obstet. Gynaec. 106 (1999) 1246–1254.
[27] K. Koga, A. Takada, N. Nemoto, Dynamic light scattering and dynamic viscoelasticity of poly(vinyl alcohol) in aqueous borax solutions. 5. Temperature effects, Macromolecules 32 (1999) 8872–8879.
[28] W.H. Eaglstein, Moist wound healing with occlusive dressings: a clinical focus, Dermatol. Surg. 27 (2001) 175–181.
[29] I. Gilko-Kabir, B. Yagen, A. Penhasi, A. Rubinstein, Phosphated crosslinked guar for colon-specific drug delivery I. Preparation and physiochemical characterisation, J. Control. Release 63 (2000) 121–127.
[30] A.F. Jimenez-Kairuz, D.A. Allemandi, R.H. Manzo, Mechanism of lidocaine release from carbomer–lidocaine hydrogels, J. Pharm. Sci. 91 (2002) 267–272.
[31] J. Ruiz, A. Mantecon, C. Cadiz, Investigation of the loading and release in PVA-based hydrogels, J. Appl. Polym. Sci. 85 (2002) 1644–1651.
[32] Y. Nitzan, A. Balzam-Sudakevitz, H. Ashkenazi, Eradication of *Acinetobacter baumannii* by photosensitized agents in vitro, J. Photochem. Photobiol. B. 42 (1998) 211–218.
[33] F. Gad, T. Zahra, T. Hasan, M.R. Hamblin, Effects of growth phase and extracellular slime on photodynamic inactivation of Gram-positive pathogenic bacteria, Antimicrob. Agents Chemother. 48 (2004) 2173–2178.
[34] Meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (TMP) product data sheet, Frontier Scientific Europe Ltd., Carnforth, Lancashire, UK, 2004.
[35] P.G. Bowler, B.I. Duerden, D.G. Armstrong, Wound microbiology and associated approaches to wound management, Clin. Microbiol. Rev. 14 (2001) 244–269.
[36] D.V. Seal, R.J. Hay, K.R. Middleton, (Eds.), Skin and Wound Infection Investigation and Treatment in Practice, Martin Dunitz Ltd., London, 2000.